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Detection of DNA adducts of acetaldehyde in peripheral white blood cells of alcohol abusers

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The effect of alcohol drinking on the formation of DNA adducts of acetaldehyde, the primary oxidative metabolite of ethanol, was investigated in humans. DNA was isolated from granulocytes and lymphocytes from 24 alcoholic patients and 12 control subjects. DNA adduct levels were measured by ^{32}P-postlabelling using reversed-phase HPLC with on-line detection of radioactivity. A large interindividual variation in adduct levels was observed. The average adduct levels in granulocyte and lymphocyte DNA from alcoholic patients were 3.4 ± 3.8 and 2.1 ± 0.8 adducts/10^7 nucleotides (n = 24), respectively. These levels were 13- and 7-fold higher than the corresponding levels in control subjects (P<0.001). The average adduct level in granulocyte DNA from alcoholic patients was 60% higher than in lymphocyte DNA (P< 0.01). Our results, in conjunction with the genotoxicity of acetaldehyde, thus suggest the formation of DNA adducts of acetaldehyde as a plausible mechanism explaining the involvement of alcohol drinking in carcinogenesis.

Introduction

Alcohol abuse is a major single cause of health impairment worldwide contributing to 15–30% of all hospital admissions in many general hospitals (1–3). Moreover, alcohol drinking is second in importance only to smoking as a proven cause of cancer (4). The evidence obtained so far shows that alcohol drinking is causally related to cancers of the mouth, pharynx, larynx, esophagus and liver (4–10). These facts, combined with high cancer risk in occupations associated with high alcohol consumption, low risk in social groups that abjure alcohol, and reduced risks in ex-drinkers, strongly suggest that alcohol abuse is carcinogenic in humans. The exact mechanisms by which alcohol abuse causes cancer in humans have not yet been determined. However, all types of alcoholic beverages seem to be implicated, suggesting an etiological role for ethanol and/or its primary oxidative metabolite acetaldehyde (Aa*).

Aa is ubiquitous in the environment (11–16). Some exogenous sources include many compounds undergoing metabolic oxidative de-ethylation such as saturated hydrocarbons (11), tobacco smoke (15), and automobile emissions (16). Moreover, endogenous formation occurs during normal metabolism from sources such as threonine, β-alanine and deoxyribose phosphate (17).

Even without any alcohol consumption the concentrations expelled in breath span from 0.2–0.6 nM, with high levels observed in smokers and abstinent alcoholics (17). More importantly, Aa is formed as the primary product during the metabolic oxidation of ethanol by alcohol dehydrogenase (ADH) in the liver (14) and causes many of the pathophysiological effects related to alcohol (1). Aa is readily oxidized further to acetate by aldehyde dehydrogenase (ALDH) (1,18). Blood and tissue concentrations of alcohol and Aa are thus dependent on the rate of alcohol and Aa oxidation. ADH and ALDH are polymorphic in humans. Individuals (mainly Orientals) who harbour an inactive aldehyde dehydrogenase variant (ALDH2*2) have high blood Aa levels upon drinking, with striking consequences in terms of facial flushing and ethanol intolerance, which in some cases have resulted in a relative aversion to alcohol, lowering consumption and the related morbidity (1,18).

Aa is a carcinogen in animal models (1,19,20) and is mutagenic to bacteria and mammalian cells (11–13,21,22). After chronic alcohol consumption, blood concentration of Aa was increased (14,17,18,23), raising the possibility that alcohol via Aa may increase the risk of the liver and extrahepatic tissues (4).

Aa is a highly reactive electrophile which readily reacts with nucleophilic groups, such as amino and sulfhydryl groups of proteins to form adducts (24–27). Furthermore, Aa is capable of interacting covalently with DNA leading to the formation of DNA adducts (28–31), a critical initiating event in the multistage process of chemical carcinogenesis. The major stable adduct formed after reduction under physiological conditions is N^2-ethyl-2′-deoxyguanosine (31,32).

Taking into consideration the biological activity of Aa and its occurrence, we suggested that the genotoxic action of Aa constitutes an important mechanism underlying the carcinogenicity associated with the ingestion of alcoholic beverages (32). We recently developed a ^{32}P-postlabelling assay using reversed-phase HPLC with on-line detection of radioactivity which proved to be sensitive enough for the quantitative determination of N^2-ethyl-2′-deoxyguanosine adducts of Aa in liver DNA from mice exposed ethanol (32).

This is the first study reporting on the detection of N^2-ethyl-2′-deoxyguanosine adducts of Aa in humans. To this end lymphocyte and granulocyte DNA isolated from peripheral blood samples of alcoholic patients and control subjects (teetotallers and moderate drinkers) were analysed for adducts of Aa by the ^{32}P-postlabelling technique.

Materials and methods

Subjects

The subject population comprised 12 control healthy individuals and 24 alcoholic patients. Control subjects were eight males and four females. The age of the individuals in the control group was 25–46 years, with a median

*Abbreviations: Aa, acetaldehyde; dG, 2′-deoxyguanosine; 3′-dGMP, 2′-deoxyguanosine-3′-monophosphate; N^2-ethyl-3′-dGMP, N^2-ethyl-2′-deoxyguanosine-3′-monophosphate adduct; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; PEI, polyethyleneimine; MN, micrococcal nuclease; SPD, spleen phosphodiesterase.

age of 32 years. This group included six teetotallers and six moderate drinkers (<5 units of alcohol a week; one UK unit is equivalent to 10 g of pure ethanol). Individuals in the control group were non-smokers, with the exception of one moderate smoker (<10 cigarettes/week). Alcohol abusers were patients admitted to the Alcohol Clinic of the Huddinge University Hospital, Stockholm, Sweden, for detoxification and rehabilitation treatment. This group included 19 males and five females, of ages 31–64, with a median age of 46 years. Alcohol intake in this group was estimated to >50 units alcohol (UK) per week. The subjects in the alcoholic group were also heavy smokers (>20 cigarettes/day). The study protocol was approved by the ethics committee of the Huddinge University Hospital.

Chemicals

All chemicals and biochemicals were of analytical grade and used without further purification, and have been described elsewhere (32).

Blood sampling and isolation of DNA

Blood was drawn by venipuncture into EDTA-containing tubes and immediately placed on ice. In the case of alcoholic patients, the blood sample was collected immediately after admission, at which time the subjects clearly exhibited symptoms of alcohol intoxication. Blood (15 ml) was diluted to 30 ml with phosphate-buffered saline (PBS), pH 7.4, and was carefully layered over 15 ml Ficoll Paque (Pharmacia Chemical Co., Uppsala, Sweden) and centrifuged at 500 *g* for 30 min at 20°C. The mononuclear fraction of white blood cells (mainly lymphocytes) on top of the Ficoll Paque layer was collected and washed twice with PBS. The cell layer below the Ficoll Paque (granulocytes and erythrocytes) was treated with 30 ml of 0.1 M NaCl, 0.5% Triton X-100, 10 mM Tris–HCl, pH 7.4, on ice for 10 min to lyse the erythrocytes. The pellet containing granulocytes was washed twice with PBS. DNA was isolated from lymphocytes and granulocytes by digestion with RNase A, RNase T1 and proteinase K, followed by extraction with phenol, chloroform/isoamyl alcohol, and precipitation with ethanol (33).

The DNA thus obtained was dissolved in distilled water and its concentration and purity were assayed using a Beckman DU 640 spectrophotometer. The ranges of the A_{230}/A_{260} and A_{260}/A_{280} in the DNA samples were 0.4–0.5 and 1.6–1.8, respectively. Also the UV spectrum (220–300 nm) was taken in order to detect the presence of shoulders. RNA contamination was assayed by reversed-phase HPLC using previously described ammonium formate/methanol gradients (34), after enzymatic hydrolysis of aliquots of DNA (10 μg) to deoxynucleosides according to Martin and Garner (35). RNA contamination levels, on the basis of the concentration of adenosine in the samples were <0.1%.

At this stage, prior to any analysis work, the DNA samples were coded to conceal their identity.

DNA hydrolysis, DNA adduct enrichment and measurement by reversed-phase HPLC with on-line detection of radioactivity

The preparation of DNA samples (5 μg) isolated from lymphocytes or granulocytes for the ^{32}P-postlabelling reaction was done according to the procedure of Fang *et al.* (32) with the modification that the MN/SPD digested DNA solutions were then further hydrolysed at pH 5.3 by adding 1 μl of nuclease P1 solution (2.5 units/μl in 1.5 mM, $ZnCl_2$; 0.5 units/μg DNA) at 37°C for 60 min to remove normal nucleotides. The prepared material was then evaporated to dryness, and redissolved in 1 μl distilled water prior to the ^{32}P-postlabelling reaction.

The recovery of the N^2-ethyl-3′-dGMP adducts after hydrolysis with nuclease P1 was determined by a series of experiments where unmodified salmon testis DNA samples (10 μg) hydrolyzed by MN and SPD were mixed with 50 fmol of authentic N^2-ethyl-3′-dGMP standard synthesized according to Vaca *et al.* (31) and subjected to hydrolysis with nuclease P1 for different intervals up to 90 min. The adduct levels were then measured by ^{32}P-postlabelling with on-line detection of radioactivity (32). The concentration and purity of the salmon testis DNA used were assayed as described above. The highest recovery obtained was 78% after incubation with nuclease P1 for 60 min. The adduct levels obtained in the human DNA samples were corrected for losses in recovery after the enrichment procedure, and for labelling efficiency and radioactive decay.

Human lymphocyte or granulocyte DNA samples (5 μg) or samples of the N^2-ethyl-3′-dGMP standard (10 fmol) were ^{32}P-postlabelled as previously described (32). The analysis of ^{32}P-labelled Aa-DNA adducts was carried out by reversed-phase HPLC with on-line detection of radioactivity according to Fang and Vaca (32) with two minor modifications. (i) 3.0 units of nuclease P1 per μg DNA were used for the conversion of 3′,5′-bisphosphates of N^2-ethyl-2′-deoxyguanosine adducts to N^2-ethyl-5′-dGMP adducts. (ii) A gradient of methanol (solvent A) in 0.2 M ammonium formate containing 20 mM phosphoric acid, pH 4.5 (solvent B) was used for elution of the adducts as follows: 0–10 min, 0% A, isocratic; 10–35 min, 0–10% A; 35–45 min, 10% A, isocratic; 45–54 min, 10–22% A; 54–57 min, 22–100% A; 57–65 min, 100% A, isocratic (programme I). The adducts were quantitated by using a calibration curve (peak area vs adduct concentration) obtained through the analysis of known amounts of the adduct standard. Normal deoxynucleosides in the enzymatic hydrolysate were quantitated by reversed-phase HPLC as described above at 254 nm with the 166 UV detector. The exact amount of parent deoxynucleosides in individual samples was calculated using a standard ratio generated from known amounts of salmon testis DNA, and used to correct the adduct level detected in DNA samples.

The efficiency of the phosphorylation reaction of N^2-ethyl-3′-dGMP adducts by T4 polynucleotide kinase was determined for each experiment using labelled N^2-ethyl-3′-dGMP standard (10 fmol) and analyzing the samples by TLC (determined as 3′,5′-bisphosphate) and by reversed-phase HPLC (determined as N^2-ethyl-5′-dGMP) as described above.

The limit of detection of the reversed-phase HPLC method with on-line detection of radioactivity was established through a series of experiments where aliquots of unmodified salmon testis DNA samples (5 μg) hydrolyzed by MN and SPD were mixed with 0.5, 1.0, 2.0, 5.0 and 10.0 fmol of N^2-ethyl-3′-dGMP standard, labelled as described above, and thereafter subjected to 3′-dephosphorylation with nuclease P1 and analysis by reversed-phase HPLC. Only background radioactivity was observed at the retention time corresponding to that of the standard when labelled salmon testis DNA samples alone were analyzed. The lowest detection limit so far achieved was 5 adducts/10^8 nucleotides, using 5 μg DNA samples, and considering all adduct losses during the preparation of the samples and analysis by ^{32}P-postlabelling.

Identification of DNA adducts of Aa detected in alcoholic patients

Two experimental schemes were used to confirm the identity of the adducts detected in alcoholic patients. For this purpose DNA isolated from granulocytes was used.

In the first scheme, a combination of TLC and reversed-phase HPLC was used to analyse the adducts as 3′,5′-bisphosphates. DNA was enzymatically digested, ^{32}P-postlabelled and resolved using two dimensional TLC-chromatography according to previously described methods (32), except that chromatography was carried out at 4°C. The adduct spot migrating at the same position as that of the bisphosphate of the N^2-ethyl-3′-dGMP standard on TLC was excised and extracted with 400 μl of 3.5 M ammonium formate, pH 3.5, at room temperature for 30 min. This procedure ensured the extraction of ~95% of the radioactivity present in the spot. This solution was then centrifuged and the supernatant was passed through an Ultrafree-MC filter (10 000 NMWL regenerated cellulose membrane, Millipore Co., Milford, MA) and the filtrate evaporated to dryness under vacuum. The sample was then re-dissolved in water and an aliquot (25 μl) of the ^{32}P-labelled extract was subjected to reversed-phase HPLC as described above. The elution was carried out with a gradient between methanol (solvent A) and 0.2 M ammonium formate containing 20 mM phosphoric acid, pH 4.5 (solvent B) as follows: 0–5 min, 0–5% A; 5–15 min, 5–30% A; 15–20 min, 30% A, isocratic; 20–25 min, 30–100% A; 25–35 min, 100% A, isocratic; 35–40 min, 100–0% A (programme II).

In the second scheme, a different reversed-phase HPLC system was used and the adducts analysed as 5′-monophosphates. The [^{32}P]-HPLC system consisted of 600 E multisolvent delivery system with a 484 Tunable absorbance detector (both Waters Chromatography). Radioactivity was measured on-line with an A 280 radioactivity detector using a 0.5 ml TRLSC-cell and scintillation fluid Flo-Scint IV (Radiomatic Instruments & Chemical Co., Tampa, FL). The analytical system had a pre-column NewGuard™ holder housing a reversed-phase 18.7 μm cartridge (Brownlee Laboratories, Santa Clara, CA). Two DeltaPak™ 5 μm C18–100 A, 150×3.9 mm columns (Waters, Millipore Co., Milford, MA) connected in series as described elsewhere (36) were used for the analysis of the adducts. DNA samples were enzymatically digested, ^{32}P-postlabelled and enzymatically converted to 5′-monophosphate DNA adducts of Aa as described above. The DNA adducts were eluted with linear gradients of acetonitrile (solvent A) in 2 M ammonium formate, 0.4 M formic acid, pH 4.5 (solvent B) as follows: 0–70 min, 0–35% A (programme III) or 0–30 min, 0% A, isocratic; 30–100 min, 0–35% A (programme IV). The flow rate was 0.5 ml/min.

Statistical analysis

The statistical analysis of the data was performed using the two-tailed Student's *t*-test for two samples assuming equal variances, the Student's *t*-test for paired means, analysis of variance (ANOVA) and correlation analysis.

Results

Identification of DNA adducts of Aa detected in alcoholic patients

The structures of Aa and of the N^2-ethyl-3′-dGMP, used as a synthetic standard adduct, are shown in Figure 1.

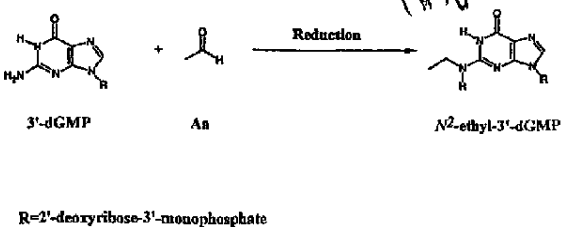

Fig. 1. Reaction scheme for the formation of N^2-ethyl-3′-dGMP adducts.

An adduct peak showing identical retention time to that of the corresponding standard was found with both experimental schemes used for the identification of the adducts as described in Materials and methods. The retention time of 3′,5′-bisphosphates of N^2-ethyl-2′-deoxyguanosine adduct was 13.0 min in the first scheme, whereas in the second the retention times of N^2-ethyl-5′-dGMP were 36.4 min and 65.2 min, with programme III and programme IV respectively (data not shown). The identity of the adduct peaks was further confirmed by analysing aliquots of the DNA samples spiked with the corresponding labelled standard in both experimental schemes.

DNA adducts of Aa in humans

Representative reversed-phase HPLC chromatographic profiles of the analysis of ^{32}P-postlabelled DNA adducts of Aa in human DNA are shown in Figure 2. The profiles were essentially identical for both granulocyte and lymphocyte DNA samples. As can be seen, an adduct peak exhibiting an identical retention time to that obtained for the N^2-ethyl-5′-dGMP standard (42.5 min) was found in granulocyte DNA from an alcoholic patient (Figure 2A and C). In addition, and for the sake of comparison, the chromatographic profile of a granulocyte DNA sample from a control subject and the profile of the same human DNA sample shown in Figure 2C spiked with 4 fmol of the adduct standard are also shown (Figure 2B and D).

The levels of DNA adducts of Aa found in human DNA samples from individuals as well as the age and the gender of the subjects are shown in Table I. At least two replicate samples of DNA from each individual were analyzed. The coefficient of variation of the analysis method was <15%. Mean values of at least two determinations of each sample are shown for each subject. As can be seen a large interindividual variation in adduct levels in alcoholic patients was found, while adduct levels in control subjects were below the detection limit of the method i.e. <5 adducts/10^8 nucleotides. In order to carry out the statistical analysis of the data an adduct level equivalent to 2.5 adducts/10^8 nucleotides (50% of the detection limit of the analysis method) was considered for the control subjects.

The average levels of DNA adducts of Aa are given in Table II. Large standard deviations were obtained in the case of alcoholic patients, reflecting the considerable interindividual variation in adduct levels as shown in Table I. Considering all individuals (both sexes and all ages), adduct levels in granulocytes and lymphocytes from alcoholic patients were 13- and 7-fold higher, respectively, than in control subjects ($P<0.001$). The average adduct level in granulocyte DNA from alcoholic patients (all subjects) was 60% higher than in lymphocytes ($P<0.01$). A significant difference in adduct levels ($P<0.05$) between granulocytes and lymphocytes was

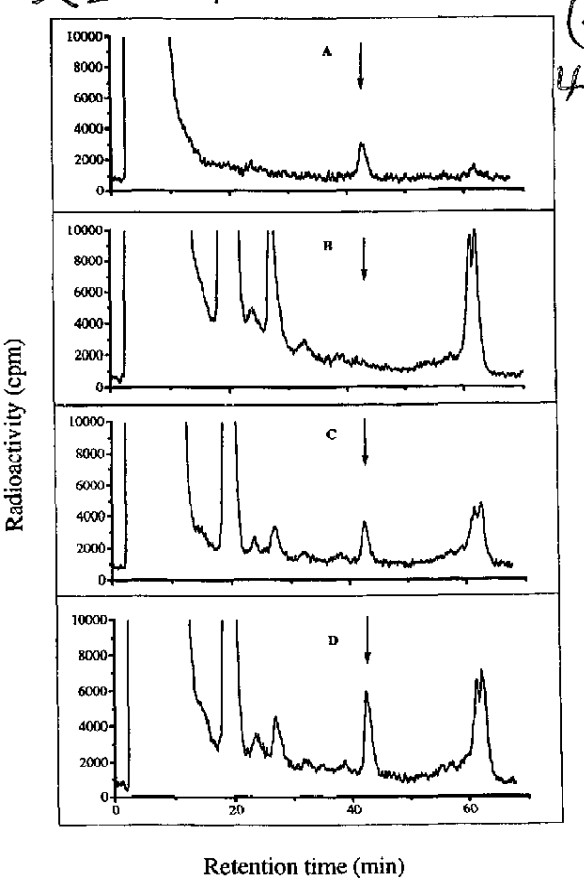

Fig. 2. Reversed-phase HPLC profiles of ^{32}P-postlabelled DNA adducts of Aa in granulocytes DNA samples. DNA was enzymatically digested with MN, SPD, nuclease P1, and analysed by reversed-phase HPLC with on-line detection of radioactivity as described in Materials and methods. (**A**) N^2-ethyl-3′-dGMP adduct standard (4 fmol); (**B**) granulocyte DNA sample from a control subject (5 μg); (**C**) granulocyte DNA sample from an alcoholic patient (5 μg); (**D**) the same DNA sample as in (*C*) spiked with N^2-ethyl-3′-dGMP adduct standard (4 fmol). The adduct peaks are indicated with an arrow.

observed only for male alcoholic patients. Further, when a correlation analysis of the adduct levels both in granulocytes and lymphocytes from the same individual was carried out, no correlation between the cell types was found.

In addition, the alcoholic patient group was divided into three age sub-groups, i.e. <40 years, 40–50 years and >50 years. The differences in adduct levels in granulocyte and lymphocyte DNA between the age categories studied were not statistically significant. Nor were sex-related statistical differences in adduct levels obtained within each cell type (Table II).

Discussion

An etiological role of alcohol consumption in cancer of the mouth, larynx, pharynx, esophagous and liver has been clearly demonstrated in several epidemiological studies (4–10). However, the molecular mechanisms underlying the carcinogenic

Table I. Levels of DNA adducts of Aa in human white blood cells, expressed as number of adducts per 10^7 nucleotides[a]

Subject no.	Age (years)	Gender	Adduct level (mean ± SD, n[b])			
			Granulocytes		Lymphocytes	
Controls						
C1	29	M	n.d.[c]	(3)	n.d.	(5)
C2	32	M	n.d.	(2)	0.3 ± 0.1	(4)
C3	32	M	n.d.	(2)	n.d	(2)
C4	40	M	n.d.	(3)	n.d	(2)
C5	33	F	n.d.	(2)	n.d	(3)
C6	46	M	n.d.	(4)	n.d	(2)
C7	29	F	n.d.	(2)	n.d	(4)
C8	33	M	n.d.	(4)	n.d	(2)
C9	25	M	n.d.	(2)	n.d	(2)
C10	30	F	n.d.	(2)	n.d	(2)
C11	32	M	n.d.	(2)	0.4 ± 0.2	(6)
C12	30	F	n.d.	(2)	n.d	(4)
Alcoholic patients						
A1	31	M	4.6±0.8	(4)	1.5±0.7	(3)
A2	56	M	n.d.	(2)	0.8±0.0	(2)
A3	41	F	0.7±0.1	(2)	2.0±0.2	(5)
A4	45	M	8.8±0.1	(2)	3.1±0.2	(2)
A5	53	M	1.7±0.7	(4)	3.9±0.6	(2)
A6	57	F	3.9±0.1	(2)	3.2±0.1	(2)
A7	59	M	2.2±0.3	(4)	2.2±0.3	(4)
A8	55	M	3.7±0.2	(2)	1.5±0.7	(4)
A9	38	M	3.7±0.2	(4)	1.9±0.2	(2)
A10	57	F	3.7±0.1	(2)	1.7±0.3	(2)
A11	31	M	3.5±0.0	(2)	3.1±0.4	(2)
A12	51	M	3.7±0.2	(2)	2.2±0.1	(2)
A13	34	F	4.4±0.4	(2)	1.6±0.4	(4)
A14	52	M	2.2±0.1	(2)	3.3±0.1	(2)
A15	33	M	1.9±0.0	(2)	2.0±1.1	(4)
A16	48	M	2.0±0.6	(4)	2.6±0.0	(2)
A17	40	M	3.4±0.3	(4)	2.8±0.1	(2)
A18	41	F	2.0±0.5	(4)	1.1±0.5	(4)
A19	36	M	2.0±0.1	(2)	1.0±0.1	(2)
A20	50	M	7.0±0.5	(2)	0.8±0.2	(4)
A21	64	M	3.5±0.5	(4)	1.4±0.2	(4)
A22	45	M	2.6±0.0	(2)	3.1±0.2	(2)
A23	37	M	2.5±1.3	(4)	1.0±0.1	(2)
A24	49	M	6.5±0.6	(2)	1.7±0.0(2)	

[a]DNA samples (5 μg) were isolated from human granulocytes or lymphocytes and the adduct levels analysed by ^{32}P-postlabelling and reversed-phase HPLC as described in Materials and methods.
[b]n = number of analyses.
[c]n.d.: Not detected. The statistical analysis was carried out considering n.d. as 50% of the detection limit of the analysis method, i.e., 0.25 adducts/10^7 nucleotides.

Table II. Difference in DNA adduct levels of Aa in peripheral white blood cells between alcoholic patients and control subjects[a]

Group	Adduct levels						Difference
	Controls			Alcoholic patients			
	mean±SD	(n)[b]	[Range]	Mean±SD	(n)[b]	[Range]	
Granulocytes							
All subjects	n.d.[c]	(12)		3.4±3.8	(24)	[n.d.–8.8]	$P<0.001$
All males	n.d	(8)		3.5±4.3	(19)	[n.d.–8.8]	$P<0.001$
All females	n.d.	(4)		2.9±2.4	(5)	[0.7–4.4]	$P<0.05$
Lymphocytes							
All subjects	0.26 ± 0.01	(12)	[n.d.–0.4]	2.1±0.8	(24)	[0.8–3.9]	$P<0.001$
All Males	0.26 ± 0.01	(8)	[n.d.–0.4]	2.1±0.9	(19)	[0.8–3.9]	$P<0.001$
All Females	n.d.	(4)		1.9±0.6	(5)	[1.1–3.2]	$P<0.01$

[a]Adduct levels are expressed as number of adducts per 10^7 nucleotides. DNA samples (5 μg) were isolated from human granulocytes or lymphocytes and the adduct levels analysed by ^{32}P-postlabelling and reversed-phase HPLC as described in Materials and methods.
[b]n = number of subjects. Parallel samples (2–6) of DNA of each individual were analysed.
[c]n.d.: Not detected. The statistical analysis was carried out considering n.d. as 50% of the detection limit of the analysis method, i.e., 0.25 adducts/10^7 nucleotides.

effects of the ingestion of alcoholic beverages remain poorly understood.

Ingested ethanol is rapidly absorbed from the gastrointestinal tract and readily metabolized to Aa (10). Considering the genotoxic properties of Aa and its endogenous formation after the ingestion of ethanol, the formation of DNA adducts of Aa was proposed as an important mechanism explaining the carcinogenicity of the ingestion of alcoholic beverages (31,32). However, the determination of such adducts was precluded by the lack of the appropriate methodology until recently, when a ^{32}P-postlabelling assay was developed in our laboratory and allowed the detection of DNA adducts of Aa formed *in vivo* in mice exposed to ethanol in the drinking water (32).

The present study demonstrates for the first time the formation of DNA adducts of Aa in humans. Alcohol abusers were considered the relevant group in this first human study since it has been shown that continuous heavy drinking leads to a faster oxidation of ethanol and/or to a slower disposition of the Aa formed with higher blood concentrations of Aa present in alcoholics than in moderate drinkers as a consequence (17,23,37).

A large interindividual variation with regard to levels of N^2-ethyl-3′-dGMP adducts (range n.d.–8.8 and 0.8–3.9 adducts/10^7 nucleotides in granulocyte and lymphocyte DNA, respectively) was found in our study (Table I). Multiple factors such as the extent of exposure, genetic polymorphism of the ADH and ALDH enzymes involved in the detoxification of ethanol and acetaldehyde respectively (14), and thus the dose of Aa to which the individuals were exposed, may explain such variation. However, no information about these factors was available for the subjects considered in this study.

As shown in Table II, higher (7- to 13-fold) and statistically significant levels of DNA adducts of Aa were found both in granulocyte and lymphocyte DNA from alcoholic patients than in control subjects. In fact no adduct levels were detected in the control individuals (Table I). If present, adduct levels in these subjects were below the detection limit of the method, i.e. <5 adducts/10^8 nucleotides. Thus, the ratios between adduct levels in alcoholic patients and control subjects should be considered as minimum ratios.

Individuals in the control group were non-smokers, with the exception of one moderate smoker (<10 cigarettes/week). On the other hand, the subjects in the alcoholic group were also heavy smokers (>20 cigarettes/day). Since Aa is also present in tobacco smoke (15), it could be argued that exposure through smoking might have contributed to the adduct levels in alcoholic subjects. However, no DNA adducts of Aa were detected in either the moderate smoker in our control group, or in granulocyte and lymphocyte DNA obtained from five heavy smokers (>20 cigarettes/day) participating in a 'quit-smoking' programme (data not shown).

No correlation between adduct levels in lymphocyte DNA and granulocyte DNA was observed. Furthermore, levels of Aa adducts in granulocyte DNA were significantly higher than in lymphocyte DNA when considering all subjects (P<0.01) or only males (P<0.05) in the group of alcoholic patients (Table II). This is in contrast to other studies (38,39), which showed that 7-methyl-guanine and polycyclic aromatic hydrocarbon (PAH)-related DNA adduct levels in lymphocyte DNA are significantly higher than those in granulocyte DNA. As the white cells are composed mainly of granulocytes (50–75%) with a short half-life (from 7 h to 2 days) and T-lymphocytes (~80% of the total lymphocytes) with a half-life of several years (40), lymphocyte adduct levels could be expected to accumulate for a chronic exposure. Blood samples from alcoholic patients were collected after acute alcohol intoxication. Therefore the adduct levels most probably reflect recent exposure. In seven alcoholic patients higher DNA adduct levels of Aa in lymphocytes than in granulocytes were detected (Table I). Besides, the samples were coded prior to the analysis to conceal their identity. Therefore methodological factors favouring the detection of adducts in granulocytes can be excluded. At present, we do not have a clear-cut explanation for the dissimilarities in adduct levels between granulocytes and lymphocytes. However, there are differences between these cell types with regard to redox potential to stabilize the N^2-ethyl-3′-dGMP adducts *in vivo* and the capacity to repair DNA damage, which would ultimately determine the adduct levels. Thus, a well functioning redox system has been described only for granulocytes (41). On the other hand, it has been shown that nucleotide and base excision repair exist in human blood lymphocytes and granulocytes. However, the repair is carried out faster and to a greater extent in lymphocytes than in granulocytes (42,43), and furthermore, the activity of uracil-DNA glycosylase in granulocytes is only 2–3 % of that in human blood lymphocytes (42).

The effect of gender was analysed because higher levels of Aa in peripheral blood than in men have been detected in women after receiving the same dose of alcohol (44). However, gender-related differences in the DNA adduct levels of Aa were not observed within each cell type in our study. It should be pointed out however, that the number of individuals in each gender category was limited. This fact, together with the high interindividual variation observed, probably precluded us from seeing any gender- or age-related significant differences in adduct levels.

In conclusion, this is the first report showing the detection of DNA adducts of Aa in humans. Adduct formation is a critical initiating event in the multistage process of chemical carcinogenesis. Since 7- to 13-fold higher adduct levels than in control subjects were detected in alcoholic patients, our results show that DNA adduct formation was causally related to alcohol abuse and thereby suggest the formation of DNA adducts of Aa, the primary metabolite of ethanol, as an important mechanism explaining the carcinogenic effects of alcohol abuse.

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References

1. Lieber,C.S. (1988) Biochemical and molecular basis of alcohol-induced injury to liver and other tissues. *N. Engl. J. Med.*, **319**, 1639–1650.
2. Bonkovsky,H.L. (1992) Detection of alcoholism and problem drinking. Liver update. *Am. Liver Fdtn*, **5**, 1–2.
3. Scheig,R. (1991) That demon rum. *Am. J. Gastroenterol.*, **86**, 150–152.
4. Austoker,J. (1994) Reducing alcohol intake. *Br. Med. J.*, **308**, 1549–1552.
5. IARC (1988) *Monograph on the Evaluation of the Carcinogenic Risk to Humans: Alcohol Drinking*. International Agency for Research on Cancer, Lyon, France, vol. 44, pp. 1–416.

6. Duffy,S.W. and Sharples,L.D. (1988) Alcohol and cancer. In Duffy,J.C. (ed.), *Alcohol and Illness*. Edinburgh University Press, Edinburgh, pp 64–127.
7. Anderson,P., Cremona,A., Paton,A., Turner,C. and Wallace,P. (1993) The risk of alcohol. *Addiction*, **88**, 1493–1508.
8. Miller,A.B., Berrino,F., Hill,M., Pietinen,P., Riboli,E. and Wahrendorf,J. (1994) Diet in the aetiology of cancer: a review. *Eur. J. Cancer*, **30A**, 207–220.
9. Blot,W.J. (1992) Alcohol and cancer. *Cancer Res.*, **52**, 2119s–2123s.
10. Longnecker,M.P. (1995) Alcohol consumption and risk of cancer in humans: an overview. *Alcohol*, **12**, 87–96.
11. IARC (1985) *Monograph on the Evaluation of the Carcinogenic Risk of Chemicals to Humans: Allyl Compounds, Aldehydes, Epoxides and Peroxides*. International Agency for Research on Cancer, Lyon, France, vol. 36, pp. 101–132.
12. Dellarco,V.L. (1988) A mutagenicity assessment of acetaldehyde. *Mutat. Res.*, **195**, 1–20.
13. Feron,V.J., Til,H.P., de Vrijer,F., Woutersen,R.A., Cassee,F.R. and van Bladeren,P.J. (1991) Aldehydes: occurrence, carcinogenic potential, mechanism of action and risk assessment. *Mutat. Res.*, **259**, 363–385.
14. Lieber,C.S., Baraona,E., Leo,M.A. and Garro,A. (1987) Metabolism and metabolic effects of ethanol, including interaction with drugs, carcinogens and nutrition. *Mutat. Res.*, **186**, 201–233.
15. IARC (1986) *Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Humans: Tobacco Smoking*. International Agency for Research on Cancer, Lyon, France, vol. 38, pp. 83–126.
16. Watson,A.Y., Bates,R.R. and Kennedy,D. (eds) (1988) *Air Pollution, The Automobile, and Public Health*. National Academy Press, Washington, DC.
17. Jones,A.W. (1995) Measuring and reporting the concentration of acetaldehyde in human breath. *Alcohol Alcoholism*, **30**, 271–285.
18. Eriksson,C.J.P. (1987) Genetic aspects of the relation between alcohol metabolism and consumption in humans. *Mutat. Res.*, **186**, 241–247.
19. Woutersen,R.A., Appelman,L.M., van Garderen-Hoetmer,A. and Feron,V.J. (1986) Inhalation toxicity of acetaldehyde in rats. III. Carcinogenicity study. *Toxicology*, **41**, 213–231.
20. Feron,V.J., Kruysse,A. and Woutersen,R.A. (1982) Respiratory tract tumours in hamsters exposed to acetaldehyde vapour alone or simultaneously to benzo[*a*]pyrene or diethylnitrosamine. *Eur. J. Cancer Clin. Oncol.*, **18**, 13–31.
21. He,S.-M. and Lambert,B. (1990) Acetaldehyde-induced mutation at the *hprt* locus in human lymphocytes *in vitro*. *Environ. Mol. Mutagenesis*, **16**, 57–63.
22. Singh,N.P. and Khan,A. (1995) Acetaldehyde genotoxicity and cytotoxicity in human lymphocytes. *Mutat. Res.*, **337**, 9–17.
23. von Wartburg,J.P. (1987) Acute aldehyde syndrome and chronic aldehydism. *Mutat. Res.*, **186**, 249–259.
24. Gapstur,S.M., de Master,E.G., Potter,J.D., Belcher,J.D. and Gross,M.D. (1992) The formation of stable acetaldehyde-hemoglobin adducts in a red blood cell model. *Alcohol*, **99**, 563–569.
25. Nicholls,R., de Jersey,J., Worrall,S. and Wilce,P. (1992) Modification of proteins and other biological molecules by acetaldehyde: adduct structure and functional significance. *Int. J. Biochem.*, **24**, 1899–1906.
26. Worrall,S., de Jersey,J., Nicholls,R. and Wilce,P. (1993) Acetaldehyde/protein interactions: are they involved in the pathogenesis of alcoholic liver disease. *Dig. Dis.*, **11**, 265–277.
27. Niemelä,O. (1993) Acetaldehyde adducts of proteins: diagnostic and pathogenic implications in disease caused by excessive alcohol consumption. *Scand. J. Clin. Lab. Invest.*, **53** (Suppl. 213), 45–54.
28. Hemminki,K. and Suni,R. (1984) Sites of reaction of glutaraldehyde and acetaldehyde with nucleosides. *Arch. Toxicol.*, **55**, 186–190.
29. Fraenkel-Conrat,H. and Singer,B. (1988) Nucleoside adducts are formed by cooperative reaction of acetaldehyde and alcohols: Possible mechanism for the role of ethanol in carcinogenesis. *Proc. Natl Acad. Sci. USA*, **85**, 3758–3761.
30. Austin,J., Dosanjh,M.K. and Fraenkel-Conrat,H. (1993) Further studies of the mixed acetals of nucleosides. *Biochimie*, **75**, 511–515.
31. Vaca,C.E., Fang,J.-L. and Schweda Elke,K.H. (1995) Studies of the reaction of acetaldehyde with deoxynucleosides. *Chem.-Biol. Interactions*, **98**, 51–67.
32. Fang,J.-L. and Vaca,C.E. (1995) Development of a ^{32}P-postlabelling method for the analysis of adducts arising through the reaction of acetaldehyde with 2′-deoxyguanosine-3′-monophosphate and DNA. *Carcinogenesis*, **16**, 2177–2185.
33. Phillips,D.H., Hemminki,K., Alhonen,A., Hewer,A. and Grover,P.L. (1988) Monitoring occupational exposure to carcinogens: detection by ^{32}P-postlabelling of aromatic DNA adducts in WBC from iron foundry workers. *Mutat. Res.*, **204**, 531–541.
34. Vaca,C.E., Vodicka,P. and Hemminki,K. (1993) Determination of malonaldehyde-modified 2′-deoxyguanosine-3′-monophosphate and DNA by ^{32}P-postlabelling. *Carcinogenesis*, **14**, 705–708.
35. Martin,C.N. and Garner,R.C. (1987) The identification and assessment of covalent binding *in vitro* and *in vivo*. In Snell,K. and Mullock,B. (eds) *Biochemical Toxicology. A Practical Approach*. IRL Press, Oxford, pp. 109–126.
36. Möller,L., Zeisig,M. and Vodicka,P. (1993) Optimization of an HPLC method for analyses of ^{32}P-postlabeled DNA adducts. *Carcinogenesis*, **14**, 1343–1348.
37. Nuutinen,H., Lindro,K.O. and Salaspuro,M. (1983) Determinants of blood acetaldehyde level during ethanol oxidation in chronic alcoholics. *Alcohol. Clin. Exp. Res.*, **7**, 163–168.
38. Mustonen,R. and Hemminki,K. (1992) 7-methylguanine levels in DNA of smokers' and non-smokers' total white blood cells, granulocytes and lymphocytes. *Carcinogenesis*, **13**, 1951–1955.
39. Savela,K. and Hemminki,K. (1991) DNA adducts in lymphocytes and granulocytes of smokers and nonsmokers detected by the ^{32}P-postlabelling assay. *Carcinogenesis*, **12**, 503–508.
40. Lotspeich-Steininger,C.A., Stiene-Martin,E.A. and Koepke,J.A. (1992) *Clinical Hematology. Principles, Procedures and Correlations*. J.B. Lippincott Company, Philadelphia, Pennsylvania.
41. Green,T.R. and Pratt,K.L. (1990) Detection and isolation of the NADPH-binding protein of the NADPH:O_2 oxidoreductase complex of human neutrophils. *J. Biol. Chem.*, **265**, 19324–19329.
42. Lankinen,M.H., Vilpo,L.M. and Vilpo,J.A. (1996) UV- and γ-irradiation-induced DNA single-strand breaks and their repair in human blood granulocytes and lymphocytes. *Mutat. Res.*, **352**, 31–38.
43. Timmerman,A.J., Mars-Groenendijk,R.H., van der Schans,G.P. and Baan,R.A. (1995) A modified immunochemical assay for the fast detection of DNA damage in human white blood cells. *Mutat. Res.*, **334**, 347–356.
44. Fukunaga,T., Sillanaukee,P. and Eriksson,C.J.P. (1993) Occurrence of blood acetaldehyde in women during ethanol intoxication: preliminary findings. *Alcohol Clin. Exp. Res.*, **17**, 1198–1200.

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